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Synthesis and *in vitro* anticancer evaluation of some 4,6-diamino-1,3,5-triazine-2-carbohydrazides as Rad6 ubiquitin conjugating enzyme inhibitors

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Abstract— Series of 4-amino-6-(arylamino)-1,3,5-triazine-2-carbohydrazides (**3a-e**) and N'-phenyl-4,6-bis(arylamino)-1,3,5-triazine-2-carbohydrazides (**6a-e**), for ease of readership, we will abbreviate our compound names as “new triazines”, have been synthesized, based on the previously reported Rad6B-inhibitory diamino-triazinylmethyl benzoate anticancer agents TZ9 and 4-amino-N'-phenyl-6-(arylamino)-1,3,5-triazine-2-carbohydrazides. Synthesis of the target compounds was readily accomplished in two steps from either *bis*-aryl/aryl biguanides via reaction of phenylhydrazine or hydrazinehydrate with key 4-amino-6-*bis*(arylamino)/(arylamino)-1,3,5-triazine-2-carboxylate intermediates. These new triazine derivatives were evaluated for their abilities to inhibit Rad6B ubiquitin conjugation and *in vitro* anticancer activity against several human cancer cell lines: ovarian (OV90 and A2780), lung (H1299 and A549), breast (MCF-7 and MDA-MB231) and colon (HT29) cancer cells by MTS assays. All the 10 new triazines exhibited superior Rad6B inhibitory activities in comparison to selective Rad6 inhibitor TZ9 that was reported previously. Similarly, new triazines also showed better

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IC₅₀ values in survival assays of various tumor cell lines. Particularly, new triazines **6a-c**, exhibited lower IC₅₀ (3.3 to 22 µM) values compared to TZ9.

Keywords: Ubiquitination; E2 ubiquitin conjugating enzyme; Rad6B; anticancer; triazines.

The ubiquitin-proteasome system controls the turnover of regulatory proteins involved in critical cellular processes including cell cycle progression, cell development and differentiation, apoptosis, angiogenesis and cell signaling pathways ¹⁻². This system requires the action of three enzymes: E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligase ³. Firstly, the ubiquitin is activated by the E1 activating enzyme and once it is activated, it is then transferred to E2 conjugating enzyme. The final step is the formation of an iso-peptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin (carried by E2). This step usually requires the action of an E3 ubiquitin ligase ⁴.

Interference with the proteasome activity was proven to be effective in cancer therapeutics since the clinical approval of bortezomib (Velcade®) as a proteasome inhibitor for treatment of relapsed multiple myeloma and mantle cell lymphoma ⁵. However, the requirement for more specific inhibiting targets like the design of potential E2 or E3 inhibitors, has appeared in order to reduce the side effects resulting from bortezomib ⁶. Recently, many E1 and E3 ligase inhibitors such as PYR-41, Nutlin-3a, P013222 and SCF-I2 have been successful and progressed to preclinical/clinical development. Also, the approved myeloma drug thalidomide has been recently identified as an E3 ligase inhibitor ⁷.

Among the E2 ubiquitin conjugating enzyme family, Rad6B is of special interest since it is found to be over-expressed in many human cancer cell lines and tumors ⁸⁻⁹. Constitutive over-expression of Rad6B in the non-transformed human breast epithelial cell line MCF 10A induces a number of adverse effects associated with cancer progression such as formation of multinucleated cells, centrosome amplification, abnormal mitosis, aneuploidy, and transformation ¹⁰. Most importantly, Rad6B has been shown to positively regulate β -catenin stabilization and activity that drives the malignant progression of breast cancer cells ¹¹⁻¹³. Since β -catenin-mediated signaling has been implicated in many human malignancies, including lung, colon, breast, and ovarian, it has been an important therapeutic target. Furthermore, Rad6B plays a central role in regulation of multiple DNA repair pathways through its interactions with different E3 ubiquitin ligases. For example, Rad6 partners with the E3 ubiquitin ligase Rad18 and monoubiquitinates PCNA in response to replication fork-stalling lesions to promote trans-lesion synthesis (TLS) or the DNA damage tolerance pathway ¹⁴⁻¹⁷. Rad18/Rad6 ubiquitin ligase complex is also important in the activation of the Fanconi anemia tumor suppressor pathway, which plays critical roles in genome integrity and tumor resistance to a variety of chemotherapeutic agents, including those that induce DNA crosslinks and DNA double strand breaks ¹⁷⁻¹⁸. Rad6 has also been shown to associate with RNF168 to monoubiquitinate histone H1.2 thereby enabling chromatin relaxation and allowing DNA damage response factors access to damage sites ¹⁹. Moreover, increased expression or activation of these **DNA damage response (DDR)** signaling and repair genes accounts for tumor resistance to chemotherapy ^{9, 20-22}. Therefore, development of DNA damage response and repair signal inhibitors are important to effectively treat these tumors.

We have recently reported [4-amino-6-(arylamino)-1,3,5-triazin-2-yl]methyl 4-nitrobenzoates TZ8-TZ9 (Fig. 1) as novel and selective Rad6B-inhibitory lead compounds²³. These inhibitors were identified by virtual screening of a pharmacophore model generated from the conserved key residues stabilizing the E2-ubiquitin thioester intermediate against a pre-prepared database using drug-like filters which determined the substituted diamino-triazine core structure as a starting point for analogue synthesis. Triazine analogue synthesis coupled to *in vitro* anticancer evaluation led to the identification of lead compounds TZ8-TZ9²³.

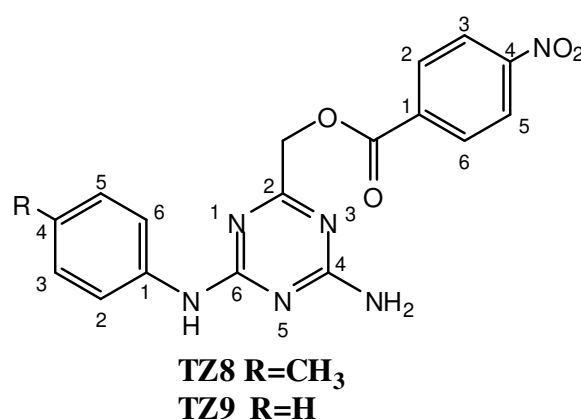


Fig.1: Chemical structures of Rad6B-inhibitory lead compounds TZ8 and TZ9

Using a molecular modeling approach to guide the design of new derivatives of TZ8 and TZ9, we reported 4-amino-N'-phenyl-6-(arylamino)-1,3,5-triazine-2-carbohydrazides (Fig. 2) with IC₅₀ values (2.48–4.79 μM) superior to those of TZ8 and TZ9 when tested on the Rad6B-expressing MDA-MB-231 cell line²⁴. In docking studies, such triazinecarbohydrazide derivatives were found to be incorporated deep inside the Rad6B binding pocket, making key interactions between the hydrazine nitrogen atoms and the Rad6B active site residues Cys88 and Asp90. Additional interactions between the aniline nitrogens and Asn119/Gln93 and between the phenyl (hydrazide) ring and Leu89 were also apparent from our docking analysis. The importance of these active

site residues to the allosteric effect on Rad6B induced by E3 ligases, and the observation that no other E2 family members (with the exception of Rad6A) have residues corresponding to Gln93 or Asn119, suggest that these triazinecarbohydrazides could be selective Rad6B inhibitors ²⁴.

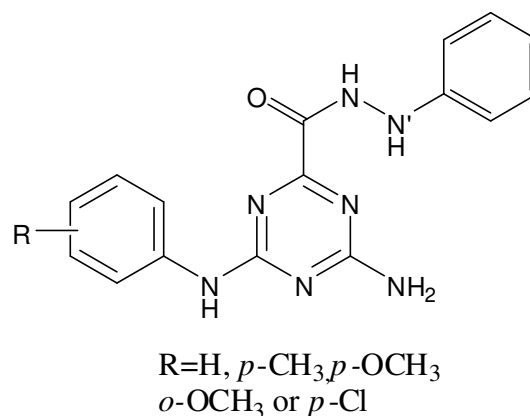


Fig.2: 4-amino-N'-phenyl-6-(arylamino)-1,3,5-triazine-2-carbohydrazides

In the current work, we studied the effect of removal of the phenyl (hydrazide) ring (**3a-e**) or the addition of aryl substituent to the free amino group (**6a-e**) of our previously reported 4-amino-N'-phenyl-6-(arylamino)-1,3,5-triazine-2-carbohydrazides (Fig. 2) on the Rad6B inhibitory activity and cellular anticancer activity. (Fig. 3)

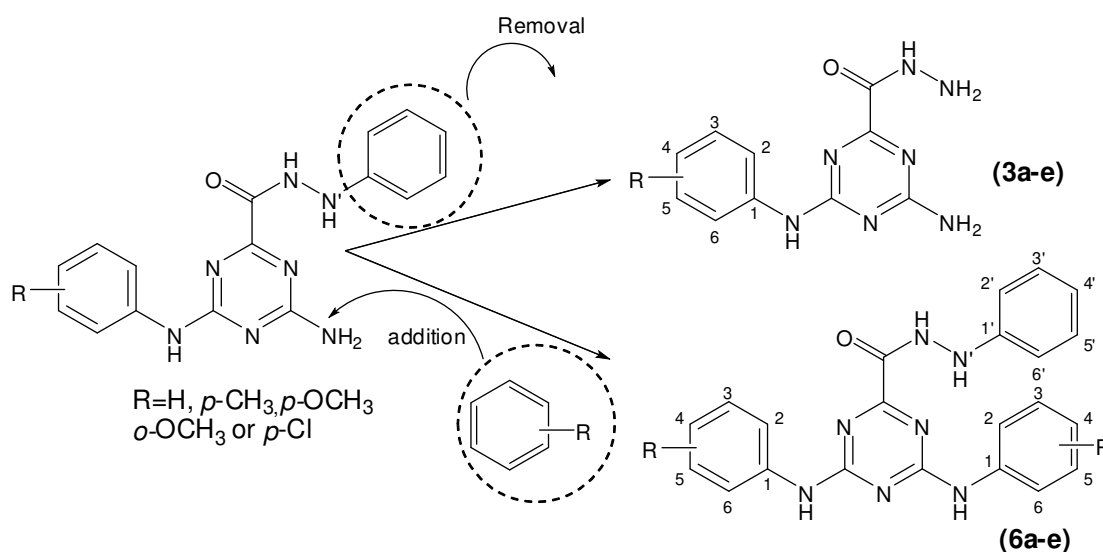
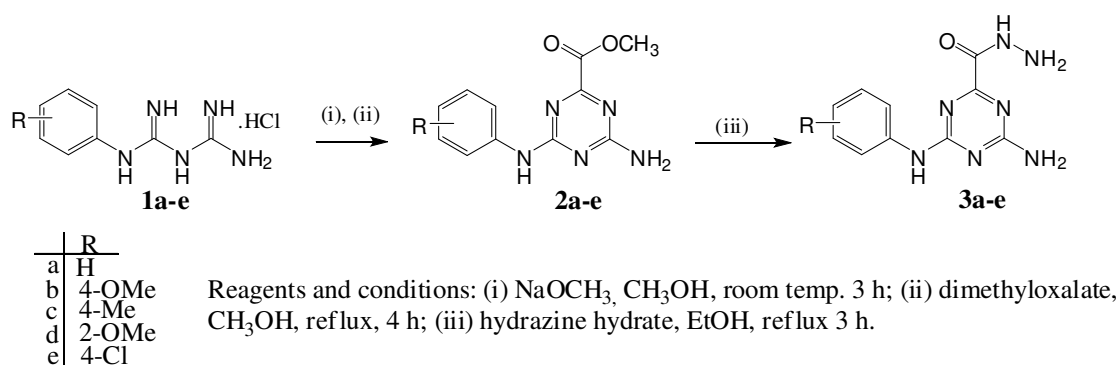


Fig. 3: SAR modifications of previous lead compounds

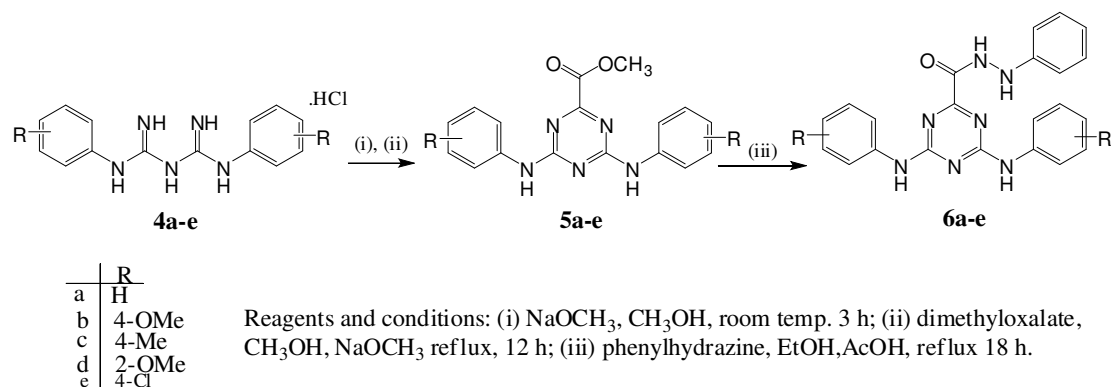
The synthesis of the 4-amino-6-(arylamino)-1,3,5-triazine-2-carbohydrazide (**3a-e**) was accomplished in two steps from arylbiguanide hydrochloride salts (**1a-e**), which were prepared from commercially available substituted aniline and dicyandiamide according to previously reported procedures²⁵⁻²⁶. Neutralization of the arylbiguanide hydrochloride salt using sodium methoxide/methanol was followed by reaction with dimethyloxalate in refluxing methanol. This gave the intermediate methyl 4-amino-6-(arylamino)-1,3,5-triazine-2-carboxylates (**2a-e**) in 83–92% isolated yield following recrystallization from methanol. Reaction of intermediates (**2a-e**) with hydrazinehydrate in refluxing ethanol produced the target new triazines (**3a-e**) in high yield (81–92%) following recrystallization from ethanol (Scheme 1).



Scheme.1: Synthetic pathway for compounds **3a-e**

A similar strategy was used to synthesize the N'-phenyl-4,6-bis(arylamino)-1,3,5-triazine-2-carbohydrazides (**6a-e**) from *bis*-arylbiguanide hydrochloride salts (**4a-e**), which were prepared from commercially available substituted aniline and sodium dicyanamide according to previously reported procedures²⁵⁻²⁶. Neutralization of the *bis*-arylbiguanide hydrochloride salt using sodium methoxide/methanol was followed by reaction with dimethyloxalate in refluxing methanol. This produced the intermediate methyl 4,6-bis(arylamino)-1,3,5-triazine-2-carboxylates (**5a-e**) in 72-87% isolated yield following recrystallization from methanol. Reaction of intermediates (**5a-e**) with phenylhydrazine in

refluxing ethanol, catalyzed by glacial acetic acid produced the target new triazines (**6a-e**) in good yield (76-86%) following recrystallization from ethanol: water (3:1) (Scheme 2).



Scheme.2: Synthetic pathways for compounds **6a-e**

Evaluation of newly synthesized 4-amino-6-(arylamino)-1,3,5-triazine-2-carbohydrazide (**3a-e**) and N'-phenyl-4,6-bis(arylamino)-1,3,5-triazine-2-carbohydrazide (**6a-e**) was carried out in the human cancer cell lines OV90 and A2780 (ovarian), H1299 and A549 (lung), MCF-7 and MDA-MB231 (breast), and HT29 (colon) using MTS assay reagent (Promega) to assess the cell viability (Table 1). TZ9 was used as a positive control and aqueous DMSO as a negative control.

To assess the efficacy of these compounds in cell cultures, MTS assays were performed using multiple tumor cell lines from different tissue origins. We have randomly chosen these cell lines as many of these tumors were previously shown to either over express Rad6 or β -catenin mediated signaling. The IC₅₀ values for each of the compounds against the individual cell line were calculated from their respective dose response curves. As shown in Table 1, almost all the compounds inhibited survival of cancer cells, although their IC₅₀ concentrations and specificities to the cell lines varied. For example, compounds **3a-e**, **6a-e** and TZ9 showed IC₅₀ values in the low micromolar range (3.3–16 μ M) when tested on HT-29 and MCF-7 cell lines, whereas only compounds **6a-d** show low micromolar IC₅₀

values (3.6–12 μM) when tested on OV 90 and A2780 cell lines. These values compare favorably with the studies on TZ9. The compound **6b** shows the lowest IC_{50} value (5 μM) when tested on the H1299 cell line. However, the reasons for these biological selectivities to particular cell lines and differences in IC_{50} values of the compounds need further evaluation. Moreover, these variations could be attributed to differences in functional groups of these compounds and inherent molecular signatures of the tumor cells, which may include their Rad6B status and their inherent dependence on its mediated signaling networks.

Name of Compound	Formula Weight	OV 90	A2780	MCF-7	MDA-MB231	A549	H1299	HT-29
TZ9	366.3	60	7.8	5	4.6	7.2	45	8.3
3a	245.24	75	7.3	5.9	2.6	7.8	65	3.3
3b	275.27	80	14.7	6.2	7.6	5.2	65	5.4
3c	259.27	60	5.5	16	12.7	9.3	40	4.5
3d	275.27	90	5.6	11.8	15.7	7.5	50	8.6
3e	279.69	85	36.6	8.8	24.6	7.5	30	4.6
6a	397.43	8	7.1	6	2.5	14.6	11	9.5
6b	457.48	12	6.3	7.2	4.2	10.8	5	5.8
6c	425.49	5	3.6	4.2	3.5	11.6	22	5.2
6d	457.48	10	5	7.2	3.9	6.9	26	8.9
6e	466.32	60	8.2	4.2	3.5	7.8	15	4.1

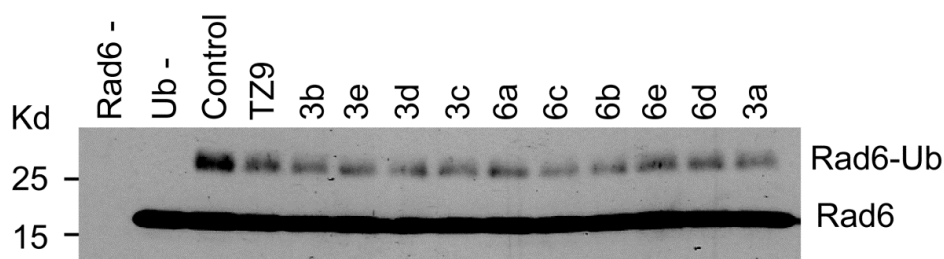
Table 1: IC_{50} Values for New Triazine Analogues.

As described in the methods, the MTS assay was used to evaluate cell survival in the presence of each new triazine analogue in the range of 0 to 125 μM concentrations. For each cell line, the MTS assays were performed at least three independent experiments and each time in triplicates. The results presented in the table are average values from multiple experiments. The IC_{50} values were calculated from the data of % survival vs. drug concentration using Microsoft Excel 2010.

To evaluate the efficacy of newly synthesized 4-amino-6-(arylamino)-1,3,5-triazine-2-carbohydrazide (**3a-e**) and N'-phenyl-4,6-

bis(arylamino)-1,3,5-triazine-2-carbohydrazide (**6a-e**) analogues for their Rad6 inhibitory activities, *in vitro* ubiquitin conjugation assays were performed in comparison with our previously reported Rad6 inhibitor TZ9. In these assays (Fig. 4A and 4B), ubiquitin conjugation to Rad6 enzyme readily occurs in the positive control (lane 3 in Fig. 4A and lane 2 in Fig. 5A), but not in the negative controls, which lacks either Rad6 (lane 1 in Fig. 4A) or ubiquitin (lane 2 in Fig. 4A and lane 1 in Fig. 5A) in these assays. Consistent with the previous data, pretreatment of Rad6 with TZ9 inhibited its ability to conjugate with ubiquitin ²³. Interestingly, in the initial screening experiments, all the new triazines exhibited Rad6 inhibitory activity (Fig 4A and 4B). As shown in the figures (4B and 5B) most of the new triazines are better Rad6 inhibitors than TZ9 at the equimolar concentration (25 nM), for both inhibition of Rad6 conjugation to ubiquitin and its substrate H2A ubiquitination in these assays.

A



B

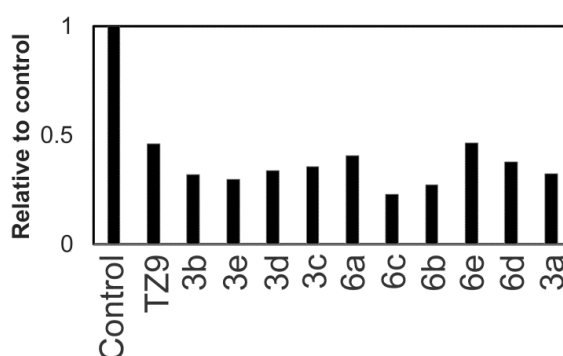


Figure 4: New triazine analogues inhibit conjugation of ubiquitin to Rad6B more effectively than TZ9. A). Representative western blot of *in vitro* ubiquitin conjugation experiment showing all new triazine analogues in comparison with TZ9 ,

and probed with Rad6 antibody. Rad6-; negative control with all experimental components except Rad6B. Ub-; negative control with all experimental components except ubiquitin. Control: positive control with all experimental components that shows Rad6B conjugation to ubiquitin. Experiment was repeated for reproducibility at a minimum of two times. B). Densitometry calculations of Rad6B-Ub bands were made using ImageJ software on the western blot shown in (A).

To further assess their inhibitory effects on Rad6B ability to transfer ubiquitin to the substrate, *in vitro* ubiquitination assays were performed using H2A as substrate ²³. We have selected four compounds **6a, b, c, e** that exhibited more favorable IC₅₀ values and better inhibitory effects on Rad6 conjugation to ubiquitin in the *in vitro* ubiquitin assays compared to TZ9. As shown in Figure 5A and 5B, compounds **6a, b, c, e** exhibited superior Rad6B inhibitory properties, both in its conjugation to ubiquitin and substrate ubiquitination (H2A-Ub) when compared to TZ9. Together, these studies report the synthesis of new triazines with better Rad6B inhibitory activities and anticancer properties compared to previously reported Rad6B inhibitor TZ9 in *in vitro* evaluations.

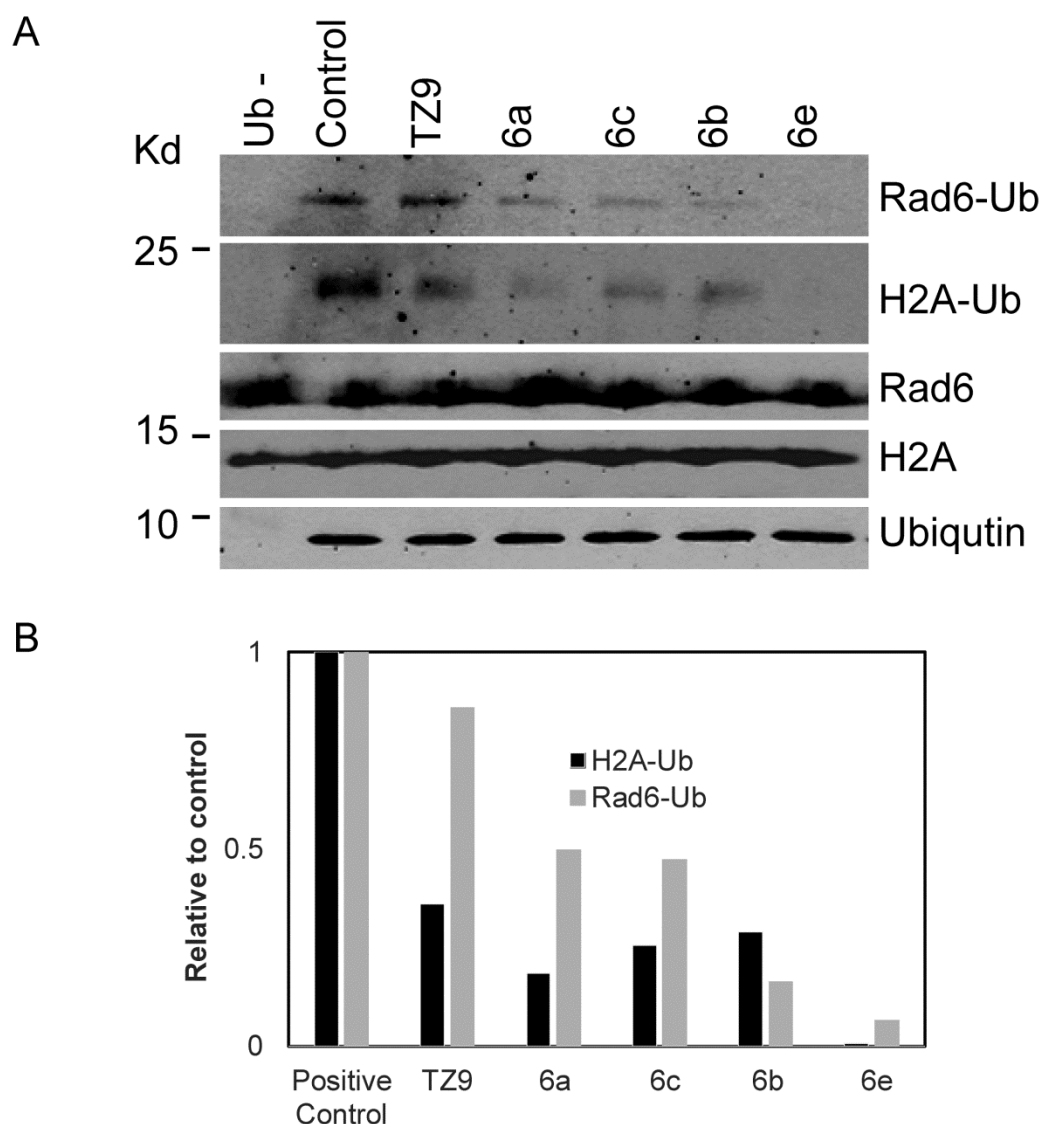


Figure 5: Rad6-mediated ubiquitination of H2A is inhibited by new triazine analogues. A). Western blot showing Rad6-mediated ubiquitination of histone H2A in the presence of new triazine analogues **probed with ubiquitin, H2A and Rad6 antibodies**. B). Represents densitometric values of western blot shown in (A), were calculated using ImageJ software and presented as relative to positive control. All experiments were repeated at least twice to verify reproducibility.

In summary, Series of novel 4-amino-6-(arylamino)-1,3,5-triazine-2-carbohydrazides (**3a–e**) and N'-phenyl-4,6-bis(arylamino)-1,3,5-triazine-2-carbohydrazides (**6a–e**) have been synthesized. Compared to the previously reported Rad6B-inhibitor TZ9, new triazines showed better IC₅₀

values in survival assays of various tumor cell lines. Particularly, new triazines **6a-c** exhibited much lower IC₅₀ (3.3 to 22 μ M) values.

Moreover, when compared to TZ9 in *in vitro* ubiquitin conjugation assays, compounds **6a, b, c, e** exhibited superior Rad6B inhibitory properties, both in its conjugation to ubiquitin and to the substrate H2A.

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Supplementary Data available:

Experimental procedure ; full compound characterisation data (m.p., ¹H, ¹³C NMR spectroscopy, mass spectrometry, % CHN analysis); In vitro ubiquitin conjugating assays and MTS assay and IC₅₀ calculations.

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